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TECHNICAL NOTE

Laboratory temperature variation is a previously unrecognized source of genotyping error during capillary electrophoresis

ANGUS DAVISON* and SATOSHI CHIBA

Division of Ecology and Evolutionary Biology, Graduate School of Life Sciences, Tohoku University, Aramaki-Aza-Aoba, Aoba-ku, Sendai 980 8578, Japan

Abstract

Capillary electrophoresis is being used increasingly for microsatellite genotyping. Here, we report that laboratory temperature greatly affects the estimated allele size, with 5 °C changing the estimated allele size by up to 0.7 bp. With accurate room temperature control, this variation would be relatively unimportant but unfortunately, a questionnaire shows that daily and seasonal laboratory temperature differences are often substantial. One solution is to use a set of reference genotypes, which also greatly assists comparisons between different platforms.

Keywords: ABI 310, capillary electrophoresis, genotyping, land snail, microsatellite, temperature

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Capillary electrophoresis (CE) is a commonly used technique for DNA genotyping, which is being used with increasing frequency for large-scale studies. As with conventional slab-gel electrophoresis, the sized alleles from a CE genotyping experiment are not discrete but instead, cluster in allelic groupings (Idury & Cardon 1997). There are several explanations for this, such as plus-A addition, but the most important factor is likely to be run-to-run variability in sizing. It is wise to design gel loading carefully, perhaps according to family relationships, and to use reference genotypes so that aberrant runs can be recognized (Ghosh *et al*. 1997). The final step in genotyping is the 'binning' of the sized alleles into discrete size classes, usually via an intermediary program such as GENOTYPER (Applied Biosystems) or ALLELOGRAM (Idury & Cardon 1997). It is absolutely critical that alleles are sized as accurately as possible prior to the binning step.

Here, we report a substantial source of error in genotyping experiments that has not previously been addressed in the literature. Commercially available genotyping platforms use a heated plate, usually at 50–60 °C, to maintain the DNA in a denatured condition (lacking secondary structure) and also to attempt to control run-to-run variation. Surprisingly, we have found that even with a heated plate, the laboratory temperature greatly affects the estimated allele size. A temperature difference of 5 °C can change the estimated allele size by up to 0.7 bp, either upwards or downwards, depending upon the allele. With accurate room temperature control this variation would matter little but unfortunately, the results of a brief survey show that daily and seasonal laboratory temperature differences are often much greater.

We used four newly isolated microsatellites from *Mandarina ponderosa*, a land snail of the oceanic Bonin Islands, to characterize the affect of variation in laboratory temperature on allele size. These microsatellites were used because they represent a range of types from short to compound dinucleotide repeats. Details of their isolation will be published in a separate primer note, along with further loci. A heterozygote individual was used in each experiment, so that the effect of temperature on four loci and eight alleles was investigated.

Denaturing CE was carried out using an ABI Prism® 310 Genetic Analyser, DATA COLLECTION Software version 1.2.2 and GENESCAN® Analysis Software version 3.1.2 (Applied Biosystems). The machine is about 2 years-old and has been recently serviced. The run module was: injection time 5 s;

Correspondence: Angus Davison. *Present address: ICAPB, Ashworth Laboratories, West Mains Road, University of Edinburgh, Edinburgh, UK EH9 3JT. Fax: (022) 217 7813; E-mail: a.davison@hgmp.mrc.ac.uk

injection voltage 15 kV; collection time 30 min; EP voltage 15 kV; heatplate temperature 60 °C; syringe pump time 240 s; preinjection EP 120 s. Microsatellites were labelled with fluorescent dye markers, either NED, 6-FAM or HEX, with the fourth dye, ROX, used for sizing (GeneScan-500 size marker, Applied Biosytems). The temperature was measured throughout the course of each run using a Thermocron iButton (Dallas Semiconductor). iButtons were placed in the laboratory, on top of the pump block within the body of the Genetic Analyser and within the anode buffer flask.

A 1 μL sample was denatured in 12 μL ultrapure formamide and 0.5 μL size marker for 120 s at 94 °C, then electrophoresed 57 times at different temperatures. Alleles were sized using the GENESCAN® local Southern method. Although global fits use more information, in practice, local estimates perform better because the relationships between size and mobility are imperfect over a wide range of sizes (Ghosh *et al*. 1997).

The temperature was varied in different runs, with the internal machine temperature approximately 1–2 °C higher than the room temperature. Figure 1 shows that a strong correlation of temperature was found with allele size, but the exact response of each locus and allele to temperature change was individual. In seven of the eight cases, the correlation was negative (Fig. 1a, c and d). In five of the eight cases, the correlation was linear (Fig. 1b, c and d), at least over the temperature range used, and in the remaining three a log curve was fitted (Fig. 1a).

The steepness of the response to temperature was initially quite surprising, as most of the capillary is kept at 60 °C throughout the run. For instance, an increase of 5 °C decreased the estimated allele size of *Mpo*8 allele 2 by around 0.8 bp, but increased the size of *Mpo*9 allele 2 by 0.4 bp (Fig. 1b). The recommendations for laboratory temperature in the ABI Prism® 310 Genetic Analyser User's Manual are that the 'ambient temperature is between 15 and 30 °C whenever the instrument is in operation, including nights and weekends', but there is no specific recommendation to maintain the same temperature at all times. If we had varied the temperature by 15 °C, then an even greater size variation might be expected. Importantly, the size variation was not due to run order, which might happen if electrophoresis was affected by capillary age (data not shown).

The increase or decrease of estimated size with temperature is relative to the size standard and thus, every fragment electrophoreses faster at elevated temperature. However, seven of the eight alleles slowed down compared with the size standard, whereas one speeded up. *Mpo*11 has a very short microsatellite, so it is not likely that this effect is due to the potentially aberrant sequence composition of long microsatellites, but is more general. The nonlinearity of the relationship between true sequence size (an integer) compared with the size standards is presumably due to differences in transient secondary structures, such as DNA bending.

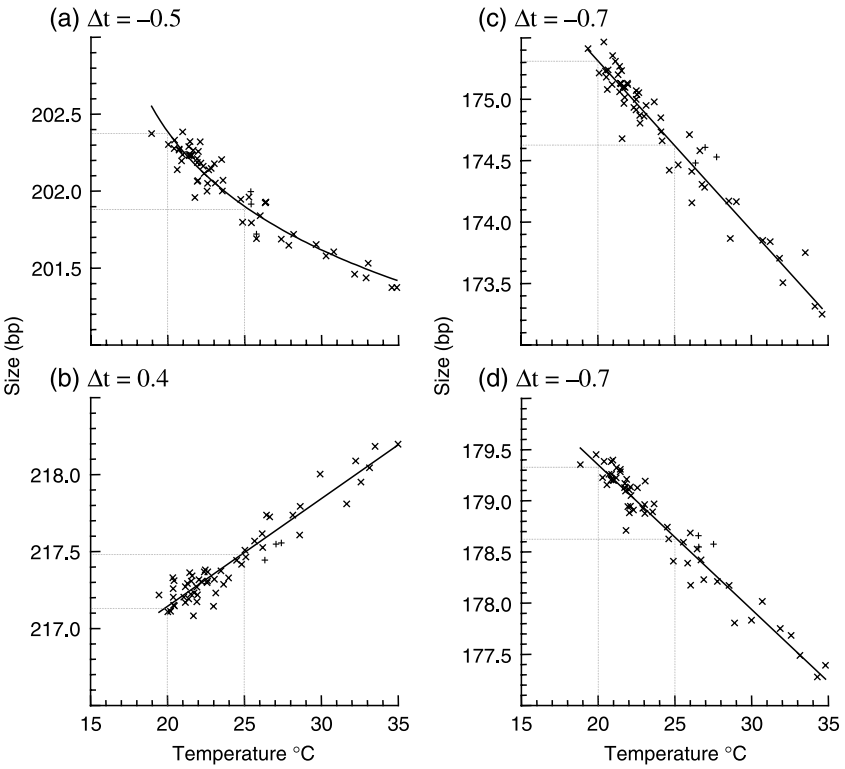

Fig. 1 Correlation of internal (block) machine temperature with estimated allele size for two microsatellite loci. Laboratory temperature is 1–2 °C lower than internal temperature. The points marked by a + are the estimated sizes, using chilled buffer, plotted against internal temperature. (a) *Mpo*9 allele 1 and (b) 2. (c) *Mpo*11 allele 1 and (d) 2. For both alleles of *Mpo*8, the relationship was similar to (a), whereas for *Mpo*10 it was like (c) and (d).

We attempted to investigate whether the problem is specific to CE machines. The variation in size could be due to a combination of buffer temperature, block temperature (containing POP-4 polymer) and the protruding ends of the capillary (approximately 3 cm). It is not possible to regulate the block temperature during a run, so we altered the temperature of the buffer either by using chilled buffer, or by placing a small beaker of ice under the anode buffer tank. This was carried out three times, interspersed with three runs using room temperature buffer. The buffer temperature throughout the run was measured via an iButton and the mean value recorded. If buffer temperature is the cause of the problem, then chilled buffer should massively change the estimated size.

In Fig. 1, the + marks indicate the estimated sizes using chilled buffer, plotted against the internal machine temperature rather than the buffer temperature. Size estimations for all three runs differed slightly compared with the main experiment, but the effect of buffer temperature was not great. The measured buffer temperatures were 21, 19 (chilled buffers) and 6 °C (ice), so allele size variation is probably a result of temperature changes in the block or protruding capillary ends. The sizes obtained using non-chilled buffer were as expected from the previous runs (not shown). Further investigations with other CE machines, such as the ABI Prism® 3100/3700 and MegaBACE system (Molecular Dynamics, USA), are necessary to establish the extent of the problem on other platforms.

To understand whether the room temperature variation is likely to be a significant cause of genotyping error, we sent a questionnaire to two listservs of primarily academic users (brian@life.biology.mcmaster.ca; saspring@sfu.ca to subscribe). The results were surprising because most machines are subject to great changes in operating temperature. Of 24 respondents, 21 (88%) thought that machine temperature control was adequate, yet the minimum mean room temperature was 17.3 °C (range = 10–25) and the maximum was 28.0 °C (range = 18–40), with the normal operating temperature 21.5 °C (range = 16–27). Eighty-three percent and 92% of users reported daily and seasonal temperature variation, respectively. The mean difference between the maximum and minimum temperatures was 10.5 °C (range = 0–22). Of the eight respondents who considered the laboratory to be a controlled temperature room, six reported that variation was still within the 2–5 °C range. It should be noted that only a few of these respondents were using capillary machines.

It is clear that the scope for error caused by room temperature variation is large. The long-term solution is to match investment in research equipment with investment in infrastructure, specifically accurate room temperature control. This could be coupled with improvements to the hardware, so that the cause of the problem can be isolated and the whole internal temperature controlled.

In the short term, several groups have developed strategies to work around the problem of variation in size, between runs and platforms. Probably, the best strategy is to use a set of reference genotypes. On an ABI Prism® 310, it would be necessary to run them about four times per 24 h if there is diurnal temperature variation (accounting for about 2 h run time); otherwise, once per day may be enough. LaHood *et al.* (2002) recently described a method for creating locus-specific ladders, which were designed for comparing data between platforms but which would be especially useful in accounting for variation due to temperature. Such a method could be used in combination with a randomised loading strategy. In this way, two genetically closely-related populations could be scored together so that temperature variation should only increase noise rather than lead to a systematic error. It would also be wise to monitor machine temperature and reject samples outside a predetermined range.

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